REVIEW

Biopsy-proven acute cellular rejection as an efficacy endpoint of randomized trials in liver transplantation: a systematic review and critical appraisal

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SUMMARY

Biopsy-proven acute cellular rejection (ACR) is the primary efficacy endpoint in most randomized trials evaluating immunosuppression in liver transplantation. However, ACR is not a major cause of graft loss, and a certain grade of immune activation may be even beneficial for long-term graft acceptance. Validated criteria to select candidates for liver biopsy are lacking, and routine clinical practice relies on liver tests, which are inaccurate markers of ACR. Indeed, both the agreement among clinicians to select candidates for liver biopsy and the correlation between the clinical suspicion of ACR and histological findings are poor. In randomized trials evaluating immunosuppression protocols, this concern grows exponentially due to the open-label and multicenter nature of most studies. Therefore, *biopsy-proven ACR* is a suboptimal efficacy endpoint given its limited impact on prognosis and the heterogeneous diagnosis, which may increase the risk of bias. Chronic rejection and/or graft loss would be more appropriate endpoints, but would certainly require larger studies with prolonged surveillances. An objective method to select candidates for liver biopsy is therefore urgently needed, and only severe episodes of histological ACR should be considered as potentially harmful. Emerging surrogate markers of ACR and antibody-mediated rejection require further investigation to determine their clinical role.

Transplant International 2016; 29: 961–973

Key words
acute cellular rejection, liver biopsy, liver transplantation, randomized controlled trial, transaminases

Received: 9 August 2015; Revision requested: 18 September 2015; Accepted: 18 December 2015

Introduction

Acute cellular rejection (ACR) is a frequent event early after liver transplantation (LT) which occurs in up to 40% of patients, although the rates reach 80% in series with protocol biopsies [1]. The majority of ACR episodes occur within the first year after LT. These features make ACR a very attractive efficacy endpoint for randomized controlled trials (RCTs) evaluating immunosuppression protocols, as fewer patients with shorter surveillance are needed for an adequately powered design. The derived reduction in operational costs is significant and favors the feasibility of RCTs. However, a suitable primary efficacy endpoint for RCTs

doi:10.1111/tri.12737

evaluating therapeutic interventions should gather other elements such as a well-established impact on prognosis and an objective and reproducible assessment. Indeed, the PRECIS tool (Pragmatic-Explanatory Continuum Indicator Summary), aiming to guide the design of clinical trials, describes an adequate primary outcome as "one objectively measured and clinically meaningful," although in some studies with a predominant explanatory component the primary efficacy outcome "may be a surrogate marker of a downstream outcome of interest" [2]. Existing evidence suggests that none of these criteria is met by *biopsy-proven ACR* in the LT setting. In this systematic review, the role of *biopsy-proven ACR* in LT is critically analyzed to better understand its impact on the risk of bias in RCTs evaluating immunosuppression after LT. In addition, some recommendations are made to improve the design of future RCTs.

Methods

A search on MEDLINE, Cochrane Controlled Trial Register (CENTRAL), EMBASE, and Science Citation Index databases was performed from January 2007 to September 2015, to analyze the current assessment of ACR in RCTs and in observational studies. We identified studies using the following keywords: "liver transplantation," "rejection," "immunosuppression," and "liver biopsy." Equivalent free-text terms were used, without language restrictions. Additional relevant studies published before 2007, and not reproduced more recently, were hand-searched. The search resulted in 1449 records which were categorized and screened independently by MRP and ET (differences resolved by MM). Duplicate records, reviews, studies on pediatric population, and unrelated articles were removed, resulting in 97 eligible studies including 63 observational studies and 34 RCTs (Fig. 1). Among these, 2 RCTs published as abstracts within the evaluated period were included although they did not provide information about the definition of ACR [3,4].

Analysis of *biopsy-proven ACR* as an efficacy endpoint for RCTs

Clinical relevance of ACR in LT

In the past, the main caveat after solid organ transplantation was the development of aggressive treatment-resistant rejection and subsequent graft loss. With the development of potent immunosuppressants, particularly calcineurin inhibitors (CNI), and with increasing clinical experience, rejection rates decreased significantly and survival was prolonged. Nowadays, the major causes of death after solid organ transplantation are infections, renal insufficiency, cardiovascular events, and de novo malignancies, which are not related to the transplantation per se, but strongly influenced by the exposure to immunosuppressants [5]. The relevance of rejection was diminished, but to a different extent depending on the organ considered. In renal transplantation, a single episode of ACR or antibody-mediated rejection (AMR), even if subclinical, may lead to chronic rejection and graft loss [6]. Likewise, in heart and lung transplantation, ACR is able to cause an irreversible damage to the graft [7,8]. The liver, however, is an immunologically privileged organ, probably due to its dual blood flow supply, its huge regeneration potential, its capacity to clear circulating antibodies, and the constant interaction with a wide spectrum of intestinal antigens. A positive cross-match does not represent a contraindication for LT [9], and it has been suggested, not without controversy, that the liver may confer a

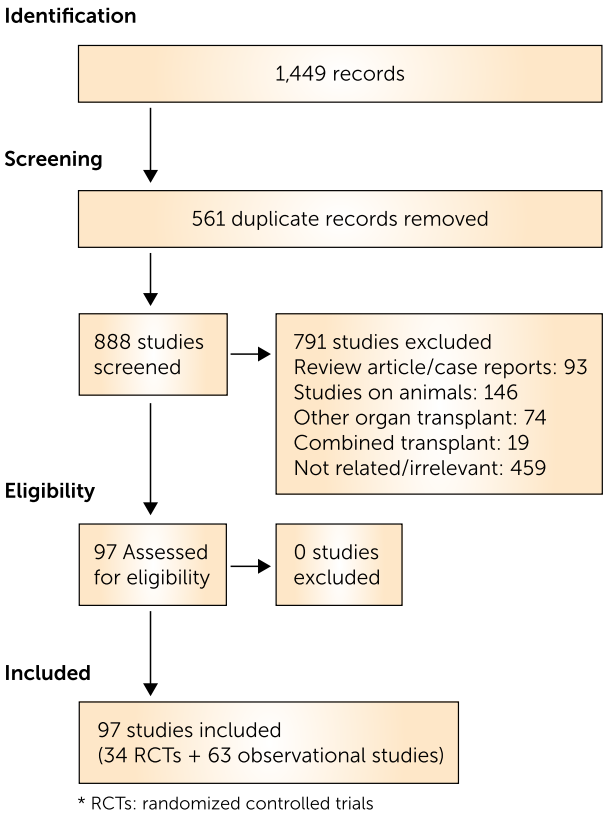

Figure 1 Flow diagram illustrating the search strategy used according to PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) statement [112].

certain protection against kidney rejection in combined organ recipients [10]. Although ACR is frequent after LT, the response to boluses of corticosteroids is successful in >80% of patients and the rates of chronic rejection remain low under current tacrolimus-based immunosuppression protocols [11]. The risk of chronic rejection and graft loss is increased in case of repeated episodes of severe ACR unresponsive to steroids and when ACR occurs late (>3 months after LT) [12–15]. Such rather rare conditions are usually restricted to a subpopulation of patients with autoimmune liver diseases [14,16]. Only 2–4% of patients will experience graft loss due to chronic rejection according to data from European and US registries [5,17].

In prolonged follow-up series with protocol biopsies early after LT, patients experiencing at least one episode of ACR, far from having an impaired prognosis, exhibit an improved long-term survival [12,18]. Even patients with moderate–severe ACR, responding to boluses of steroids, had a benefit in terms of survival when compared with patients without ACR [18]. This may be particularly true for patients without hepatitis C who will form the vast majority among the LT population in the upcoming years due to the widespread availability of the new antivirals. In light of these observations, it has been hypothesized that a certain grade of immunological insult could benefit the engraftment, while promoting operational tolerance and using minimal immunosuppression in the long term [19]. Aiming at a complete suppression of ACR at all costs, using increased exposure and number of immunosuppressants, is not only unnecessary and inconvenient after LT [20], but would adversely affect long-term outcome [21].

A recent RCT evaluating a combination of belatacept, basiliximab, mycophenolate, and steroids was prematurely terminated because of increased rates of graft loss and death compared with simpler regimes (tacrolimus±mycophenolate), although none of the deaths were attributable to ACR [22]. In another double-blind trial with protocol liver biopsies, 156 patients were randomized to tacrolimus monotherapy versus tacrolimus and steroids [23]. Although at day 7 moderate–severe histological ACR was present in almost 50% of the study population, 5-year incidence of chronic rejection was only 2.4%, while the 5-year patient and graft survival rates were excellent (76% and 79% in each arm, respectively). Thus, *biopsy-proven ACR* after clinical suspicion does not meet the criterion of "clinically meaningful" for efficacy endpoints in RCTs [2].

Selection of candidates for liver biopsy after LT

Although ACR graded following Banff criteria is an objective and validated outcome [24] within protocol biopsy populations [25], most LT programs have abandoned this strategy, claiming increased costs and derived complications. Overall complication rates after liver biopsy in adults are 6.7%, but major complications are infrequent (0.5%) and mortality rates are <0.1% [26,27]. Several factors related to an increased risk of ACR including (but not restricted to) younger age [28–31], vitamin D deficiency [32,33], pretransplant cardiac dysfunction [34], and autoimmune liver disease [16] have not been routinely taken into account to guide clinical decisions. Nowadays, only those patients with clinical suspicion of rejection, which usually means otherwise unexplained raising transaminases and/or cholestatic parameters, are selected for liver biopsy. This strategy, termed as *biopsy-proven ACR* after clinical suspicion, is used as the primary efficacy endpoint in most RCTs evaluating immunosuppression in LT. Indeed, from 2007 to 2015, 34 RCTs evaluating immunosuppression were published [3,4,22,35–65] (Table 1), and among them, only two studies (5.8%) implemented protocol biopsies early after LT to assess ACR [40,43]. The remaining 32 RCTs (94.2%) relied on *biopsy-proven ACR* as efficacy endpoint: in 20 studies, *biopsy-proven ACR* was the primary efficacy endpoint, either alone ($n = 14$) or as part of a composite endpoint ($n = 6$). *Biopsy-proven ACR* was kept as a secondary efficacy endpoint in the remaining 12 studies which aimed to prevent recurrence of hepatitis C ($n = 4$), to preserve renal function ($n = 6$), to minimize post-LT diabetes mellitus ($n = 1$), and to analyze pharmacokinetics ($n = 1$).

However, liver tests are neither sensitive nor specific for ACR, and there are no defined thresholds to determine whether a patient is at risk or not for ACR at a certain time point post-LT [66]. Among 30 RCTs assessing *biopsy-proven ACR* published in full between 2007 and 2015 (Table 1), 28 studies (93.3%) did not provide any criteria to select candidates for liver biopsy, and some of them even accepted a "pure" clinical diagnosis of ACR without histological evaluation [39,42,46,47,63]. The latter practice, not supported by clinical guidelines, may lead to misdiagnosis and unnecessary antirejection therapy, which has been linked to inferior graft survival [67]. In such studies without defined criteria, the decision to perform liver biopsy was left to the discretion of the responsible clinician according to the routine clinical practice from each institution, which may vary among clinicians (even

Table 1. Randomized controlled trials evaluating *de novo* immunosuppression protocols after liver transplantation published in full from 2007 to 2015. Definition of acute cellular rejection used and design features.

Author	Year	Centers (*n*)	Primary outcome	Blinding	*N*	ACR definition	Pure clinical rejection	Follow-up (months)	Treatment arms	ACR rates (%)
Trunecka [65]	2015	72	Renal function	Open label	893	Biopsy-proven no criteria defined for liver biopsy	Not allowed	6	TAC+MMF Anti-ILr2+TAC+MMF Anti-ILr2+TAC (delayed)+MMF	18 12.4 18.4
Klintmalm [22]	2014	39	Composite (BPAR, graft loss, and death)	Open label	260			12	TAC±MMF BEL_HD+MMF±anti-IL2r BEL_LD+MMF	20.6 36.9 32.7
Asrani [61]	2014	31	Composite (BPAR, graft loss, and death)	Open label	224	Biopsy-proven no criteria defined for liver biopsy	Not allowed	24	TAC+STDs SIR+TAC+STDs	30.4 26.4
Levy [62]	2014	45	HCV recurrence	Open label	356	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC±others CyA±others	11.2 15.4
Teperman [60]	2013	10	Composite (BPAR, graft loss, and death)	Open label	293	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC/CyA+MMF SIR+MMF	10.3 11.5
Takada [59]	2013	6	Composite (BPAR, recurrence hepatitis C, graft loss, death)	Open label	75	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC+STD TAC+MMF	11.4 32.5
Ramirez [58]	2013	1	BPAR	Open label	40	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	Anti-IL2r+TAC+MMF+STD Anti-IL2r+TAC+MMF	5 5
Pelletier [57]	2013	1	BPAR	Open label	100	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC+MMF+STD TAC+MMF	14 20
Fischer [56]	2012	15	Renal function	Open label	203	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC+STD EVE+STD (TAC weaning)	15.3 17.7
Ju [54]	2012	1	BPAR	Open label	82	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	Anti-IL2r+TAC+STDs Anti-IL2r+TAC+STDs (24 h avoidance)	7.3 9.8
De Simone [53]	2012	79	Composite (BPAR, graft loss, and death)	Open label	1147	Biopsy-proven no criteria defined for liver biopsy	Not allowed	24	TAC+STD EVE+STD TAC+EVE+STD	18.9 26.8 12.3
Neumann [55]	2012	8	HCV viral load	Open label	135	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC±MMF+STD Anti-IL2r+TAC±MMF	30.9 16.4

Table 1. Continued.

Author	Year	Centers (*n*)	Primary outcome	Blinding	*N*	ACR definition	Pure clinical rejection	Follow-up (months)	Treatment arms	ACR rates (%)
Fischer [63]	2011	11	Pharmacokinetics	Open label	129	Biopsy-proven no criteria defined for liver biopsy	Allowed	2	TACbd+STD TACqd+STD	27.4 26.9
Boudjema [51]	2011	7	BPAR	Open label	195	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC+STD TAC+MMF+STD	46 30
Masetti [49]	2010	1	Renal function	Open label	78	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	Anti-IL2r+CyA+STD Anti-IL2r+EVE+STD	7.7 5.7
Calmus [48]	2010	14	Renal function	Open label	199	Biopsy-proven no criteria defined for liver biopsy	Not allowed	24	TAC+MMF+STD Anti-IL2r+TAC+MMF+STD	25.1 24.5
Trunecka [50]	2010	48	BPAR	Double blinded	471	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TACbd+STD TACqd+STD	26.9 29.5
Benítez [47]	2010	1	BPAR	Open label	37	Biopsy-proven no criteria defined for liver biopsy	Allowed	12	TAC+STD ATG+TAC (weaning)	31.2 66.7
Nashan [44]	2009	15	Composite (BPAR and graft loss)	Open label	60	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC+MMF+STD Reduced TAC+MMF+STD	17 17
Manousou [43]	2009	3	Composite (recurrence of hepatitis C, unresponsive rejection, and graft loss)	Open label	103	Protocol biopsies at day 7	Not allowed	60	TAC+AZA+STD TAC montherapy	8.2 3.7
Boillot [64]	2009	1	BPAR	Open label	93	Biopsy-proven no criteria defined for liver biopsy	Not allowed	60	TAC+MMF+STD ATG+TAC+MMF	14.3 11.4
Neuberger [46]	2009	8	Renal function	Open label	525	Biopsy-proven no criteria defined for liver biopsy	Allowed	12	TAC+STD TAC+MMF+STD Anti-IL2r+TAC+MMF+STD	24.3 26.8 16.7
Otero [45]	2009	12	BPAR	Open label	157	Biopsy-proven no criteria defined for liver biopsy	Not allowed	6	TAC+STD Anti-IL2r+TAC+MMF	26.6 11.5
Shenoy [42]	2008	1	BPAR	Open label	60	Biopsy-proven no criteria defined for liver biopsy	Allowed	12	TAC+STD±MMF CyA+STD±MMF	27 23

Table 1. Continued.

Author	Year	Centers (*n*)	Primary outcome	Blinding	*N*	ACR definition	Pure clinical rejection	Follow-up (months)	Treatment arms	ACR rates (%)
Lupo [41]	2008	1	BPAR	Open label	47	Biopsy proven (if fever malaise, abdominal pain, or raising transaminases)	Not allowed	21	CyA+STD Anti-IL2r+CyA	28.6 15.3
Lerut [40]	2008	1	BPAR	Double blinded	156	Protocol biopsies at day 7	Not allowed	12	TAC+STD TAC monotherapy	48.7 50
Becker [39]	2008	37	BPAR	Open label		Biopsy-proven no criteria defined for liver biopsy	Allowed	3	TAC+MMF Anti-IL2r+TAC	16.2 19.7
Moench [36]	2007	1	Diabetes, dyslipidemia, hypertension	Double blinded	110	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC+STD TAC+Placebo	35.2 41.8
Vivarelli [38]	2007	2	Recurrence of hepatitis C	Open label	39	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC+STD (early withdrawal) TAC+STD (late withdrawal)	8.7 25
Schmeding [37]	2007	1	BPAR	Open label	99	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC+STD Anti-IL2r+TAC+STD	37.2 52.1
Klintmalm [76]	2007	9	BPAR	Open label	312	Biopsy-proven (if raising transaminases or bilirubin)	Not allowed	12	TAC+STD TAC+MMF+STD Anti-IL2r+TAC+MMF	35.9 36.6 30.6
Kato [35]	2007	1	Recurrence of Hepatitis C	Open label	70	Biopsy-proven no criteria defined for liver biopsy	Not allowed	12	TAC+STD±MMF Anti-IL2r+TAC±MMF	38.4 33

ACR, acute cellular rejection; anti-IL2r, anti-IL-2 receptor; ATG, antithymocyte globulin; BEL_HD, belatacept high dose; BEL_LD, belatacept low dose; BPAR, biopsy-proven acute rejection; CyA, cyclosporine; EVE, everolimus; MMF, mycofelonate; SIR, sirolimus; STD, steroids; TAC, tacrolimus.

within the same center), and may introduce a significant heterogeneity in the diagnosis of ACR. The risk of performance and detection bias is increased, but grows exponentially in the two following situations: (i) multicenter studies: More participating institutions means more clinicians involved in the decision-making process, having different practices regarding selecting candidates for liver biopsy. Among the RCTs included in Table 1, 63% were multicenter (n = 20), involving a median of 13 different institutions per study (IQR 8–38); (ii) open-label design: Nearly all RCTs in Table 1 were open-label (29 of 32; 90.6%). The clinician was aware of the immunosuppression protocol and it is possible that he would be more worried about ACR in those patients having received less potent immunosuppression protocols. In a certain patient with a mild–moderate modification of liver tests, the indication for a liver biopsy could rely on the immunosuppression protocol; thus, a patient having *a priori* more potent immunosuppression (more drugs and/or higher exposure) may avoid liver biopsy if an improvement occurs, whereas a patient under *a priori* less potent protocol (monotherapy with CNI or reduced exposure) would be more likely to undergo a liver biopsy. The full impact of these factors is difficult, if not impossible, to assess given that the rates of liver biopsy in each comparison arm are not reported in RCTs.

The agreement among clinicians to select patients with clinical suspicion of rejection was explored in a recent study including 100 LT patients with protocol biopsies and histological assessment of ACR early after LT [29]. The relevant clinical information between LT and the protocol liver biopsy including demographics, etiology of liver disease, immunosuppression, and daily liver tests was given to nine highly experienced clinicians from three transplant centers who decided whether a liver biopsy was needed on an individual case basis. The concordance among clinicians to advice liver biopsy was poor (κ<0.40 in 76% of comparisons), but even more striking was the low concordance between the "clinical suspicion of ACR" and the presence of actual features of histological ACR in the protocol liver biopsy (κ<0.30 in all cases) [29]. These findings reinforce the hypothesis that the evaluation of an objective and prospectively validated outcome as it is histological ACR assessed by the Banff criteria has been transformed into a subjective and partially evaluated outcome. Therefore, *biopsy-proven ACR* after clinical suspicion does not meet the criterion "objectively measured" for efficacy endpoints to be used in RCTs.

Recommendations to optimize the assessment of ACR within RCTs

Mild-moderate histological ACR should not be considered an adverse outcome

In clinical practice, the minimization of immunosuppression is gaining adepts [68]. In the last decade, several RCTs have evaluated protocols with reduced exposure to CNI, either by lowering their trough concentrations or by delaying their introduction. Most of these trials added new drugs such as mTOR inhibitors [53,56], mycophenolate [46,51], anti-IL2r [46,48,65], or antithymocyte globulin [47,64] to counteract the expected increased risk of ACR, and thus, many authors are of the opinion that there was not a true minimization. There is a demand for RCTs evaluating protocols with complete avoidance (or early withdrawal) of CNI or, if these drugs are to be kept, to use them as monotherapy and/or with reduced trough concentrations. These strategies may require a protocolized histological evaluation, and may be accompanied by increased rates of ACR, but most patients may experience a benefit in the long term.

In the past, several studies had to stop the CNI-free (or early withdrawal) arm due to increased *biopsy-proven ACR* rates. In the H2304 study [53], the role of everolimus as a renal sparing agent was explored, either in monotherapy (after tacrolimus withdrawal within 4 months after LT) or in combination with reduced tacrolimus. The control group received tacrolimus and steroids. The everolimus monotherapy arm was stopped due to increased rates of *biopsy-proven ACR* (19.9%) when compared with the reduced tacrolimus and everolimus arm (4.1%) and the control group (10.7%), although most ACR episodes were mild, and there were no differences in terms of graft loss/death [53]. Most patients in the everolimus monotherapy group were then converted to other immunosuppression regimes, but still in the 24-month extension study, they exhibited the best glomerular filtration rates [69]. Indeed, the PROTECT trial, which also evaluated everolimus monotherapy after tacrolimus withdrawal (within 12 months after LT), showed similar rates of *biopsy-proven ACR* in the interventional arm (17.7%), but the trial was not stopped and there was a sustained benefit on renal function at 3 years [56,70]. Another RCT evaluated antithymocyte globulin as a tacrolimus sparing agent [47], a protocol widely used in renal transplantation, even for patients at increased immunological risk [71]. Again the trial in LT patients was stopped because

of increased rates of ACR with antithymocyte globulin (52.4%) as compared with controls (25%), although most ACR episodes in the interventional arm were mild not requiring boluses of steroids. Not a single episode of steroid-resistant ACR occurred.

Despite all these evidences, histological ACR, if properly assessed, may still provide relevant information about graft allo-reactivity. The clue resides in the fact that histological ACR needs to be interpreted as a dynamic process which takes place in around 80% of patients at some point [40,66,67]. In most of these patients, there will only be mild histological changes without pathological consequences, even when they are associated with raising transaminases. In RCTs, only severe episodes of histological ACR, or moderate episodes not responding to boluses of steroids, should be considered as negative events. Mild episodes of histological ACR or moderate changes responding well to boluses of steroids should not form part of any stopping rule or efficacy endpoint in RCTs, as they do not adversely impact long-term outcome [12,18,67].

The selection of candidates for liver biopsy should be standardized

In RCTs aiming at aggressive minimization or complete avoidance of CNI, it is absolutely necessary to perform protocol liver biopsies with a central and blinded pathology reading, particularly early after LT, and this may be ethically fitting considering the potential short- and long-term benefits that these protocols may offer in terms of renal impairment [69,70,72], de novo tumors [73], recurrence of hepatocellular carcinoma [74], and graft loss [18], among others. In RCTs evaluating more conventional regimens based on CNI, a protocolized pathological surveillance may not be strictly warranted, but the criteria to select patients for liver biopsy should be standardized to ensure a homogeneous evaluation of ACR. A method to identify patients at increased risk of ACR (i.e., candidates for liver biopsy) early after LT has been seldom attempted, and never fully accomplished. In 1992, a definition of clinical suspicion of rejection based on liver tests (ALT increase >50 U/l and/or bilirubin >6 mg/dl reversed by antirejection therapy) was compared to histological findings [75]. The correlation was not good and 40% of patients biopsied had histological rejection, not encompassed by the clinical definition. The HCV3 trial [76] considered a patient at clinical suspicion of ACR whenever 3 consecutive test results revealed serum AST or ALT levels elevated 1.5 times above the baseline or serum bilirubin elevated by 0.3 mg/dl. In another study, patients were biopsied provided they had fever, malaise, back or abdominal pain, tenderness or enlargement of the liver, a change in bile color, and a rapid increase in transaminases or cholestatic parameters [41]. However, neither transaminases nor bilirubin (not to say fever, malaise or abdominal pain) has shown any diagnostic capacity of ACR in previous studies [66,77], and the chosen thresholds for liver tests (if any) were arbitrary, without any prior analysis. The actual benefit of these methods is unknown, as the rates of liver biopsy due to clinical suspicion of rejection were not reported. However, an important concept was introduced: a dynamic change on liver tests was considered more appropriate for a noninvasive suspicion of ACR, rather than static values. The above-referred study based on 100 LT patients with protocol liver biopsies early after LT explored a multivariate model to predict moderate–severe histological ACR based on the product age by pre-LT MELD, the immunosuppression protocol, and the delta blood eosinophil count within the 4 days prior to liver biopsy [29]. The area under ROC curve to predict moderate–severe histological ACR was 0.84, and it allowed to stratify patients according to the expected rate of ACR, in order to guide clinical decisions. The rates of misdiagnosis following the derived algorithm were as low as 10%. These results should be validated, and further modifications of the model explored before recommending its implementation in routine clinical practice. An international consensus is urgently needed to define what is meant by clinical suspicion of rejection after LT, and it should be based on objective, reproducible, and dynamic parameters, able to translate the events taking place in the liver graft. Only then an objective assessment of rejection will be possible within double-blinded RCTs.

Composite endpoints including *biopsy-proven ACR* after clinical suspicion should be interpreted cautiously

The doubtful prognostic impact of *biopsy-proven ACR* as currently assessed has led to the use of composite efficacy outcomes including combinations with chronic rejection, graft loss, and mortality [22,43,44,53,59–61]. In such endpoints, *biopsy-proven ACR* is a much more frequent event and will be the main (and maybe the only significant) contributor to produce outcomes. The caveat derived from using composite endpoints with a predominant component is well known, for instance, in cardiovascular trials [78], and will not allow to overcome the problem of *biopsy-proven ACR*. The use of chronic rejection, graft loss, and mortality as the only

primary efficacy endpoints would require an unbearable high number of patients with prolonged surveillances, thereby increasing costs exponentially and reducing feasibility. Nonetheless, an observational follow-up of patients included in RCTs evaluating immunosuppressants should be systematically extended at least for 5 years to report graft loss and mortality rates, as these data may reinforce the justification of the evaluated strategy [23]. A meta-analysis of individual patient data and a network meta-analysis from several RCTs using this information would be extremely valuable, particularly for those studies with interventional arms prematurely stopped due to increased early ACR rates. It is possible that these interventional arms, usually aiming at aggressive minimization or avoidance of CNI [47,53], show similar or even improved graft loss and mortality rates than more conventional immunosuppression protocols, as already reported in a long-term follow-up of a tacrolimus monotherapy RCT [23]. In that case, such aggressive minimization protocols should not be discarded, but further investigated for a possible benefit on adverse events. Regulatory authorities should consider imposing 5-year reports on chronic rejection, graft loss, and mortality in RCTs as it would help to determine the optimal immunosuppression protocol for each patient, to develop more accurate clinical guidelines, and to allow for a true tailored immunosuppression in LT.

Future directions

The long-standing promise of personalized medicine is becoming a reality. In solid organ transplantation, there is an increasing amount of immunosuppressive drugs and combinations. Ideally, a minority of LT patients at increased risk of aggressive ACR and graft loss may require intensive immunosuppression and pathological surveillance of the liver graft, whereas immune-tolerant patients would benefit from minimization strategies with an improved safety profile, and long-term weaning of immunosuppression [68]. For this purpose, the clinician needs to be provided with discriminative and not-invasive diagnostic tools. However, the search of reliable biomarkers for immune-mediated diseases is a real challenge, given the intricated mechanisms underlying the activation of the different immune pathways, which in turn use to be interconnected and protected by hidden feedback signals.

Immune function assays evaluate the response of different components of the immune system after a certain stimuli, which can be donor-antigen specific or not-antigen specific [79]. Antigen-specific assays confront stimulator cells from the donor with mononuclear cells from the recipient, to analyze the amount of cytokines produced. Although the methodology is based in a solid rationale, the need of viable donor cells (not available within deceased donation) and the lack of standardized procedures have limited its applicability. Among not-antigen-specific immune assays, the most invoked, and the only approved by Food and Drug Administration for immune monitoring, is the Immuknow test (Cylex LTD, United States), which measures the production of intracellular adenosine triphosphate by T-CD4+ cells after stimulation with phytohemagglutinin. Serial determinations after LT may predict ACR as well as consequences of over-immunosuppression such as infections. Unfortunately, the obtained results are inconsistent. A meta-analysis of five observational studies implementing Immuknow after LT ($n = 543$) found a significant heterogeneity among publications. This is not surprising as none of these studies were based on protocol liver biopsies, but instead used *biopsy-proven ACR* after clinical suspicion as the gold standard [80]. A recent RCT compared a group with standard practice (dose of tacrolimus adjusted according trough concentrations) ($n = 102$) with an interventional arm ($n = 100$) in which tacrolimus dosage was modified according to serial Immuknow determinations. The immunosuppression protocol was tacrolimus and tapering steroids in all patients, although additional immunosuppressants were permitted. The interventional arm had reduced trough concentration of tacrolimus, which resulted in less infections (42% vs. 54.9%; $P < 0.05$), with similar biopsy-proven ACR rates (19% vs. 13.7%; P = NS), and improved survival rates at 12 months (95% vs. 82%; $P < 0.01$) [81]. Although it seems that Immuknow adds some information to liver tests and trough concentrations of CNI, more studies are warranted to confirm these observations.

Flow cytometry is another powerful technique able to detect and quantify activated T cells in peripheral blood. In allo-reactive patients, the proliferation of activated T cells is an early event, providing a perfect window of opportunity to implement changes in immunosuppression. Among lymphocyte subpopulations, Th17, activated CD8+ and CD4+ T cells are increased in patients with ACR [82–85], whereas Treg cells promote tolerance [86]. Again, these results should be interpreted with caution as the gold standard used was any grade of *biopsy-proven ACR* after clinical suspicion. Other strategies based on microRNAs [87–91], mRNAs [92–94], enzyme-linked immunosorbent spots [95], serum cytokines concentrations and polymorphisms [96–99], proteomic signatures [100], and genomic fingerprints [101–103] have been tested in LT, but they are far from becoming a reality in clinical practice.

Historically, AMR has received little attention in LT as HLA-incompatible donors do not impact on long-term recipient survival [104]. However, a single episode of humoral rejection increases the risk of chronic rejection [105]. The screening of donor-specific antibodies using the luminex system is gaining adepts among kidney transplant physicians, but the actual meaning in the LT setting is still unknown. The liver has a tremendous potential to clear preformed donor-specific antibodies, and most patients will remain free from humoral rejection. It seems that the thresholds for donor-specific antibodies in liver recipients should be set higher than in renal or heart transplantation [106], but hitherto they are not established. Those patients with persistent class II HLA donor-specific antibodies after LT are at increased risk of significant rejection and graft loss [106]. However, although a lower concentration of donor-specific antibodies increases the risk of ACR, its impact on graft survival needs to be further explored [107,108]. Patients developing high titers of persistent donor-specific antibodies after LT would provide valuable additional information for RCTs, as they might explain some cases of graft loss of unknown origin [109], and some histological criteria have been established [110]. However, a more accurate and individualized phenotyping will be needed before the implementation of AMR as an endpoint in RCTs in LT [111].

Conclusion

Biopsy-proven ACR after clinical suspicion is an inappropriate efficacy outcome for RCTs evaluating immunosuppressive protocols. This shortcoming is hindering the way toward minimal immunosuppression and operational tolerance. The LT community has chosen to turn a deaf ear on this matter, but the evidence calls for a change. In RCTs using aggressive minimization regimens, protocol liver biopsies should be implemented, including a central and blinded pathology reading. For RCTs with more conventional CNI-based immunosuppression, an objective methodology to select candidates for liver biopsy is urgently needed in order to homogenize the assessment of ACR among clinicians and transplant teams worldwide. In the early post-LT period, only severe episodes of ACR, and maybe moderate episodes unresponsive to steroid boluses, should be considered as potentially harmful events. Chronic rejection and derived graft loss might be the primary efficacy outcomes to measure, and surrogate biomarkers of such events are warranted.

Funding

Dr. Manuel Rodríguez-Perálvarez is a recipient of the Physician scientist fellowship awarded in 2015 by the European Association for the Study of the Liver (EASL). Prof. Jan Lerut has an unrestricted grant from Fresenius Biotech. MR-P, JR-J, ET, PB, and MM have nothing to disclose concerning the present manuscript.

Conflicts of interest

The authors have declared no conflicts of interest.

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